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PEX13 Is Mutated in Complementation Group 13 of the Peroxisome-Biogenesis Disorders

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Summary

The peroxisome-biogenesis disorders (PBDs) are a genetically and phenotypically diverse group of diseases caused by defects in peroxisome assembly. One of the milder clinical variants within the PBDs is neonatal adrenoleukodystrophy (NALD), a disease that is usually associated with partial defects in the import of peroxisomal matrix proteins that carry the type 1 or type 2 peroxisomal targeting signals. Here, we characterize the sole representative of complementation group 13 of the PBDs, a patient with NALD (patient PBD222). Skin fibroblasts from patient PBD222 display defects in the import of multiple peroxisomal matrix proteins. However, residual matrix-protein import can be detected in cells from patient PBD222, consistent with the relatively mild phenotypes of the patient. *PEX13* encodes a peroxisomal membrane protein with a cytoplasmically exposed SH3 domain, and we find that expression of human *PEX13* restores peroxisomal matrix-protein import in cells from patient PBD222. Furthermore, these cells are homozygous for a missense mutation at a conserved position in the PEX13 SH3 domain. This mutation attenuated the activity of human *PEX13*, and an analogous mutation in yeast *PEX13* also reduced its activity. The mutation was absent in >100 control alleles, indicating that it is not a common polymorphism. Previous studies have demonstrated extragenic suppression in the PBDs, but the phenotypes of patient PBD222 cells could not be rescued by expression of any other human *PEX* genes. Taken together, these results provide strong evidence that mutations in *PEX13* are responsible for disease in patient PBD222 and, by extension, in complementation group 13 of the PBDs.

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Introduction

Zellweger syndrome (ZS; MIM 214100), neonatal adrenoleukodystrophy (NALD; MIM 202370), infantile Refsum disease (IRD; MIM 266510), and rhizomelic chondrodysplasia punctata (RCDP; MIM 215100) together comprise the peroxisome-biogenesis disorders (PBDs; Lazarow and Moser 1995). These phenotypically heterogeneous diseases can be subdivided into two groups, the Zellweger spectrum and the RCDP spectrum. Within the Zellweger spectrum, patients with ZS display the most severe phenotypes. These patients rarely survive the first year, exhibit pronounced hepatic, renal, and neurologic deficits, and display relatively severe defects in the import of all classes of peroxisomal matrix proteins. The phenotypes of patients with NALD are similar but less pronounced, and these individuals usually survive beyond the first year. Patients with IRD are the most mildly affected individuals in this group and in some instances may survive into their third or fourth decade. Zellweger spectrum diseases can be caused by defects in any of ⩾10 different genes, eight of which are known (Shimozawa et al. 1992; Dodt et al. 1995; Yahraus et al. 1996; Chang et al. 1997; Portsteffen et al. 1997; Reuber et al. 1997; Honsho et al. 1998; Warren et al. 1998; Matsuzono et al. 1999; South and Gould 1999). Phenotype-genotype studies suggest that the different clinical phenotypes in the Zellweger spectrum are due to differences in allele severity rather than to the gene that is defective (Reuber et al. 1997; Chang and Gould 1998; Collins and Gould 1999). In contrast, all patients in the RCDP spectrum fall within a single complementation group, CG11 (Braverman et al. 1997; Motley et al. 1997; Purdue et al. 1997). These patients, like those in the Zellweger spectrum, display developmental delay and multitissue dysfunction but are distinguished by numerous features, including the presence of pronounced rhizomelia and widespread epiphyseal calcifications. In addition, patients with RCDP display a much more restricted set of peroxisomal metabolic abnormalities.

The underlying cause of the PBDs is a defect in the

import of one or more classes of peroxisomal matrix proteins, a phenotype that can be caused either by defects in the peroxisomal matrix–protein import machinery or by factors that are required for synthesis of peroxisome membranes. Peroxisomal proteins are encoded by nuclear genes, contain peroxisomal targeting signals, and are imported posttranslationally (Lazarow and Fujiki 1985). Two types of peroxisomal targeting signals have been identified, PTS1 and PTS2 (Subramani 1993). PTS1 (Gould et al. 1989) is a C-terminal tripeptide of the sequence serine-lysine-leucine-COOH, or a conservative variant, and is found on the vast majority of peroxisomal proteins. PTS2 (Swinkels et al. 1991) is commonly found near the N-terminus of proteins and consists of the sequence RLX_5HL, but it explains the targeting of only a handful of peroxisomal proteins.

The receptors for PTS1 and PTS2 have been identified as the products of the *PEX5* and *PEX7* genes, respectively, and mutations in these genes are responsible for disease in complementation groups 2 and 11 of the PBDs (Dodt et al. 1995; Braverman et al. 1997; Motley et al. 1997; Purdue et al. 1997). In addition to the PTS receptors, other peroxisome-biogenesis factors have been found to be defective in the PBDs. The interacting ATPases PEX1 and PEX6 are defective in complementation groups 1 and 4 of the PBDs, the two most abundant groups within the Zellweger spectrum (Fukuda et al. 1996; Yahraus et al. 1996; Portsteffen et al. 1997; Reuber et al. 1997). Also, a trio of zinc-binding integral peroxisomal membrane proteins, PEX2, PEX10, and PEX12, have been identified as the factors defective in groups 10, 7, and 3, respectively (Shimozawa et al. 1992; Chang et al. 1997; Okumoto et al. 1998*a,* 1998*b;* Warren et al. 1998). More recently, defects in peroxisome-membrane synthesis have been identified as a cause of the PBDs, most notably in *PEX16*-defective CG9 cells (Honsho et al. 1998; South and Gould 1999) and *PEX19*-deficient cells from a 14th complementation group, CG-J (Matsuzono et al. 1999).

The PTS receptors appear to be predominantly cytoplasmic proteins and may shuttle between the cytoplasm and peroxisome as they mediate matrix-protein import (Marzioch et al. 1994; Dodt and Gould 1996). Thus, they must interact with the peroxisome at several points in the import process, the first of which is docking. PEX13, PEX14, and PEX17 have been implicated in PTS-receptor docking (Elgersma et al. 1996; Erdmann and Blobel 1996; Gould et al. 1996; Albertini et al. 1997; Huhse et al. 1998; Girzalsky et al. 1999) and may comprise a receptor-docking complex. Although candidate human *PEX13* and *PEX14* genes have been identified previously (Gould et al. 1996; Bjorkman et al. 1998; Fransen et al. 1998) there has yet to be a patient who is defective in these components of the peroxisomal protein–import apparatus. Shimozawa et al. (1998) reported the identification of a patient with NALD who defined a novel complementation group of the PBDs, CG13, the 11th independent complementation group within the PBDs. In the present study, we demonstrate that this patient is defective in the import of multiple peroxisomal matrix proteins and that this phenotype is caused by a missense mutation, I326T, in the human *PEX13* gene.

Material and Methods

Cell Lines and Antibodies

The cell line from patient PBD222 has been described previously (Shimozawa et al. 1998), as has the control human skin-fibroblast line 5756T (Chang et al. 1999). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with penicillin, streptomycin, and 10% FCS (Life Sciences). Rabbit polyclonal antibodies to PMP70 were raised against the C-terminal 18 amino acids of the protein (Gärtner et al. 1992), rabbit polyclonal antibodies to human PEX14 were generated against a fusion between PEX14 and maltose-binding protein, rabbit polyclonal anti-SKL antibodies have been described elsewhere (Gould et al. 1990), mouse anti-myc monoclonal antibodies were obtained from the tissue-culture supernatant of the hybridoma cell line 1-9E10 (Evan et al. 1985), and sheep polyclonal anticatalase antibodies were obtained from The Binding Site (San Diego). Fluorescently labeled goat secondary antibodies were obtained from commercial sources.

Plasmids

The plasmid designed to express full-length human *PEX13* (pcDNA3-*PEX13*) has been described elsewhere by Bjorkman et al. (1998). The plasmid designed to express the mutant form of *PEX13* detected in patient PBD222 cells, pcDNA3-*PEX13*/I326T, was created by excising the 953-bp *Asp*718-*Nhe*I fragment from pcDNA3-*PEX13*, excising the 300-bp *Nhe*I-*Eco*RI fragment from pCR2.1-*PEX13*/PBD222 (see Mutation Detection below), and inserting these two fragments between the *Asp*718 and *Eco*RI sites of pcDNA3 (Invitrogen). Prior reports have described the *PEX* gene–expression vectors pcDNA3-*PEX1* (Reuber et al. 1997), pcDNA3-*PEX5* (Dodt et al. 1995), pcDNA3-*PEX6* (Yahraus et al. 1996), pcDNA3-*PEX7* (Braverman et al. 1997), pcDNA3-*PEX10* (Warren et al. 1998), pcDNA3-*PEX11*α and pcDNA3-*PEX11*β (Schrader et al. 1998), pcDNA3-*PEX12* (Chang et al. 1997), and pcDNA3-*PEX16* (South and Gould 1999). The plasmids pcDNA3-*PEX2,* pcDNA3-*PEX3,* pcDNA3-*PEX14,* and pcDNA3-*PEX19* were constructed by inserting a full-length cDNA for these human cDNAs (Shimozawa et al. 1992; Braun et al. 1994; Fransen et al. 1998; Kam-

merer et al. 1998) downstream of the CMV promoter in pcDNA3. Prior reports have also described the peroxisomal marker-protein–expression vectors pcDNA3-PAHXmyc (Mihalik et al. 1997), pcDNA3-NmycPECI (Geisbrecht et al. 1999), pcDNA-NmycPTE1 (Jones et al. 1999), and pcDNA3-NmycGOX (J. M. Jones and S. J. Gould, unpublished data). The expression vector for the N-terminal myc-tagged form of human catalase (pcDNA3-NmycCatalase) was created by amplifying the open reading frame of the human catalase cDNA by PCR and cloning it downstream of the N-terminal myc-epitope tag in pcDNA3-NmycPTE1 (Jones et al. 1999).

pDC120 is a *HIS4*-based replicating vector for the yeast *Pichia pastoris* that contains the wild-type *P. pastoris PEX13* gene (Gould et al. 1996). An analogous plasmid containing a mutant *PEX13* gene that lacks activity in vivo (pDC120/E291K) has also been described (Gould et al. 1996). V334 of *P. pastoris* Pex13p is analogous to I326 of human PEX13, and pDC120 was modified to substitute a threonine for valine 334, as follows: A 535-bp fragment containing the *PpPEX13* SH3 domain-coding sequence was amplified by PCR, with pDC120 as template and the oligonucleotide primers 5′-GAAACGAATCATGGGCAAGTTGATGGG-3′ and 5′-AAACTGCAGCGGCAGGTTCTTCCTGAGCCTCAGGCACGGGTCGTTGATGTCGTTCAATGATTTCGACGTAATTGTAAGGTGTAAATCCAACCTTACCATC-3′, the latter incorporating the V334T coding-sequence change. The *Eco*RI-*Pst*I fragment from this PCR product was cloned between the *Eco*RI and *Pst*I sites of pDC120, creating pDC120/V334T. The amplified portion of this clone was sequenced to ensure the absence of any unintended mutations.

Immunofluorescence

Human fibroblasts were grown on glass cover slips until ready for analysis. The cells, still attached to cover slips, were removed from the growth medium, washed twice with Dulbecco's modified phosphate-buffered saline (DPBS; 0.2 mg/ml KCl, 0.2 mg/ml KH_2PO_4, 8 mg/ml NaCl, 2.16 mg/ml $Na_2HPO_47H_2O$, 0.11 μg/ml $CaCl_2$, and 0.95 μg/ml $MgCl_2$ [pH 7.15]), and incubated in 3.7% formaldehyde in DPBS for 15–30 min. The cells were then washed twice in DPBS and incubated in 1% Triton X-100 in DPBS for 5 min to permeabilize the cellular membranes. Next, the cells were washed twice in DPBS and then incubated with primary antibodies for 15 min. Rabbit polyclonal anti-PMP70 antibodies were used at a dilution of 1:300, rabbit polyclonal anti-PEX14 antibodies were affinity purified and used at a dilution of 1:100, rabbit polyclonal anti-SKL antibodies were used at a dilution of 1:300, and sheep polyclonal anticatalase antibodies were used at a dilution of 1:400; and the tissue-culture supernatant from 1-9E10 cells—the source of mouse anti-myc monoclonal antibody—was undiluted. All antibodies were diluted in DPBS with 0.1% BSA except for double-label experiments with anti-myc antibodies, in which case they were diluted into the 1-9E10 tissue-culture supernatant. After 10 washes with DPBS, the cells were incubated with secondary antibodies for 15 min. At the end of this incubation the cells were washed 10 times with DPBS and mounted onto glass slides in a small volume (5–10 μl) of 90% glycerol, 100 mM Tris-HCl (pH 8.5), and 0.01% *p*-phenylenediamine.

Transfections and Calculation of Relative Rescue

Cells were transfected by electroporation, as described by Chang et al. (1997), and were processed for immunofluorescence two days after transfection. The numbers of cells expressing each marker protein were counted, and each expressing cell was placed into one of three categories on the basis of the distribution of the marker protein, as follows: (1) cytoplasmic only staining, in which no peroxisomal staining could be detected; (2) cytoplasmic plus peroxisomal staining, in which the cells had significant cytoplasmic staining but also displayed peroxisomal staining; and (3) peroxisomal only staining, in which the protein appeared to be predominantly or exclusively peroxisomal. The percentages of cells displaying no import, partial import, and complete import of the peroxisomal marker protein were calculated for cells transfected with pcDNA3 (vector control), the *PEX13*-expression vector (pcDNA3-*PEX13*), and the *PEX13*/I326T–expression vector, pcDNA3-*PEX13*/I326T. Corrected rescue values were calculated by subtracting the percentage of import observed in cells transfected with vector alone from the percentage of import observed in cells transfected with the *PEX13*- and *PEX13*/I326T–expression vectors. Relative rescue was then obtained by dividing the corrected rescue values by the corrected rescue value determined for the cells transfected with the *PEX13*-expression vector. Relative complete rescue was calculated in an analogous manner except that only the cells demonstrating complete rescue (i.e., import of the marker protein into peroxisomes with little or no cytoplasmic staining) were considered.

A minimum of 500 expressing cells were characterized in each trial. All plasmids used in these experiments were of similar purity, as determined by UV-absorption spectrometry and agarose-gel electrophoresis. Furthermore, two independent preparations of the pcDNA3-*PEX13* and pcDNA3-*PEX13*/I326T plasmids were used in these experiments (one set was used for assessing the import of NmycCatalase (trial 1), NmycGOX (trials 1 and 2), NmycPTE1, NmycPECI, PAHXmyc (trial 1), and endogenous catalase; the second set of plasmids were used

for assessing the import of NmycCatalase (trials 2 and 3), NmycGOX, (trial 3), and PAHXmyc (trial 2).

Mutation Detection

We isolated genomic DNA from control skin fibroblasts (5756T cells) and from patient PBD222 fibroblasts by using the PureGene kit as recommended by the manufacturer (Gentra Systems). Genomic DNA (100 ng) was then used as a template for the amplification of the four *PEX13* exons with use of the oligonucleotides described (table 1), all of which are based on the sequence of the *PEX13* gene (Bjorkman et al. 1998). HsPEX13.1 and HsPEX13.4 were used for amplification of a 196-bp fragment containing exon 1, HsPEX13.5 and HsPEX13.6 were used for amplification of a 945-bp fragment containing exon 2, HsPEX13.7 and HsPEX13.8 were used for amplification of a 307-bp fragment containing exon 3, and HsPEX13.9 and HsPEX13.2 were used for amplification of a 393-bp fragment containing exon 4. Each PCR product was purified and sequenced directly. We also examined the structure of the *PEX13* mRNA in patient PBD222. Total RNA was isolated from control skin fibroblasts (5756T cells) and from patient PBD222 fibroblasts with the PureScript kit as recommended by the manufacturer (Gentra Systems). First-strand cDNA was synthesized from patient PBD222 RNA with a *PEX13*-specific oligonucleotide (HsPEX13.3) as a primer. The cDNA product was then used as a template for the amplification of the *PEX13* open reading frame with the *PEX13*-specific oligonucleotides HsPEX13.1 and HsPEX13.2. This *PEX13* cDNA product was cloned into the T/A vector pCR2.1 as recommended by the manufacturer's suggestions (Invitrogen). The *PEX13*-derived fragment of the resulting plasmid, pCR2.1-*PEX13*/PBD222, was sequenced in its entirety.

To determine the frequency of the *PEX13*/I326T mutation, we amplified a 199-bp fragment of the *PEX13* gene using two oligonucleotides (HsPex13.12 and HsPex13.13) that flank the I326 codon. The fragment amplified from the wild-type gene is cleaved into 164-bp and 35-bp fragments by the restriction enzyme *Bst*ZI7I, but the fragment amplified from the *PEX13* gene with the I326T mutation is resistant to digestion because the mutation eliminates the restriction-enzyme site. This fragment of *PEX13* was amplified from 51 control genomic DNA samples representing 102 *PEX13* alleles. In each case, a single PCR product of the correct size was obtained and no product was detected in control samples that lacked genomic DNA (all amplifications were carried out in sets of $\leq$16 samples, and positive and negative control samples were included in each set of reactions). The products of these reactions were then digested with *Bst*Z17I and separated by agarose-gel electrophoresis, and the relative masses of the products were determined by ethidium-bromide staining and interpolation from standards of known size. We were unable to detect undigested material from any of the 51 control samples (data not shown), indicating that they lacked the I326T mutation. This was in stark contrast to amplified material from patient PBD222 genomic DNA, which was entirely resistant to digestion with *Bst*ZI7I, as well as the amplification products from mixtures of control and patient PBD222 genomic DNA, which were only partially digested with *Bst*Z17I (data not shown).

Table 1

Oligonucleotides Used in Amplifying *PEX13* Exons and cDNA

Name	Sequence (5′–3′)
HsPex13.1	GTCAGGGGTAGGAGCGGGAGCC
HsPex13.2	GTTCAACTGCAGGCAAACATGAAAG
HsPex13.3	CCAGGTCACCAGACCAACATACTAGTG
HsPex13.4	ACCGCCTCCTGCCAAGTCC
HsPex13.5	CAGTAGAATTTGTCATAGCACCAGG
HsPex13.6	TCCTCTGCCAGTTTTGAAGCTC
HsPex13.7	GCAAGCCATAGCCAGTGAAATATC
HsPex13.8	CTCTAAGTTAACTAACAATGAACAAGATCC
HsPex13.9	CCATTACTATTCTGTTGGACCTCC
HsPex13.12	TTCTGGCTAGCCTTGATGGCCAAACAACA GGACGTA
HsPex13.13	AACAGATTCAAAGGCAGCTTCCTGTTCATCC

Yeast Studies

The *P. pastoris PEX13*-deletion mutant (*arg4*-1, *leu2*Δ::*ARG4*, *pex13*Δ::*LEU2*, *his4*Δ::*ARG4*) has been previously described by Gould et al. (1996), as have the general procedures for the growth and manipulation of *P. pastoris* strains (Crane and Gould 1994; Crane et al. 1994). The *pex13*Δ strain was transformed to histidine prototrophy with the plasmids pDC120, pDC120/E291K, and pDC120/V334T. Three independent transformants for each were precultured in minimal glucose medium lacking histidine (Crane and Gould 1994) and then transferred to minimal methanol medium lacking histidine, and the growth of each culture was monitored by following the optical density at 600nm of the cultures.

Results

Phenotypes of Patient PBD222 Fibroblasts

The 11th independent complementation group of the PBDs, CG13, is defined by a single patient with NALD (Shimozawa et al. 1998), whom we designate patient PBD222 in our patient-numbering system. Previous studies have demonstrated that the PBDs can be caused by defects in either peroxisomal matrix–protein import or the synthesis of peroxisome membranes (Santos et al. 1988; Chang et al. 1999; Matsuzono et al. 1999; South

and Gould 1999). To determine which process may be defective in patient PBD222, we examined the ability of patient PBD222 cells to import a variety of peroxisomal membrane proteins and peroxisomal matrix proteins. Patient PBD222 cells appeared to contain hundreds of peroxisomes that could be labeled with antibodies to either of two well-characterized peroxisomal membrane proteins, PMP70 (Kamijo et al. 1990) and PEX14 (Fransen et al. 1998) (fig. 1*A* and *B*). In contrast, the classic peroxisomal matrix–protein marker, catalase, was distributed throughout the cytoplasm of patient PBD222 cells (fig. 1*C*). An Nmyc-tagged form of catalase containing the 10 amino acid myc epitope at its N-terminus was introduced into patient PBD222 cells and also was found accumulated in the cells' cytoplasm rather than imported into their peroxisomes (fig. 1*D*). Catalase terminates in an atypical form of the PTS1, KANL (Bell et al. 1986; Purdue and Lazarow 1996), rather than in the prototypical PTS1 of SKL (Gould et al. 1989), and is the least efficiently imported peroxisomal matrix protein known (Lazarow et al. 1982). To test whether patient PBD222 cells were defective in import of other peroxisomal matrix proteins, we examined the distribution of two human proteins that terminate in the tripeptide

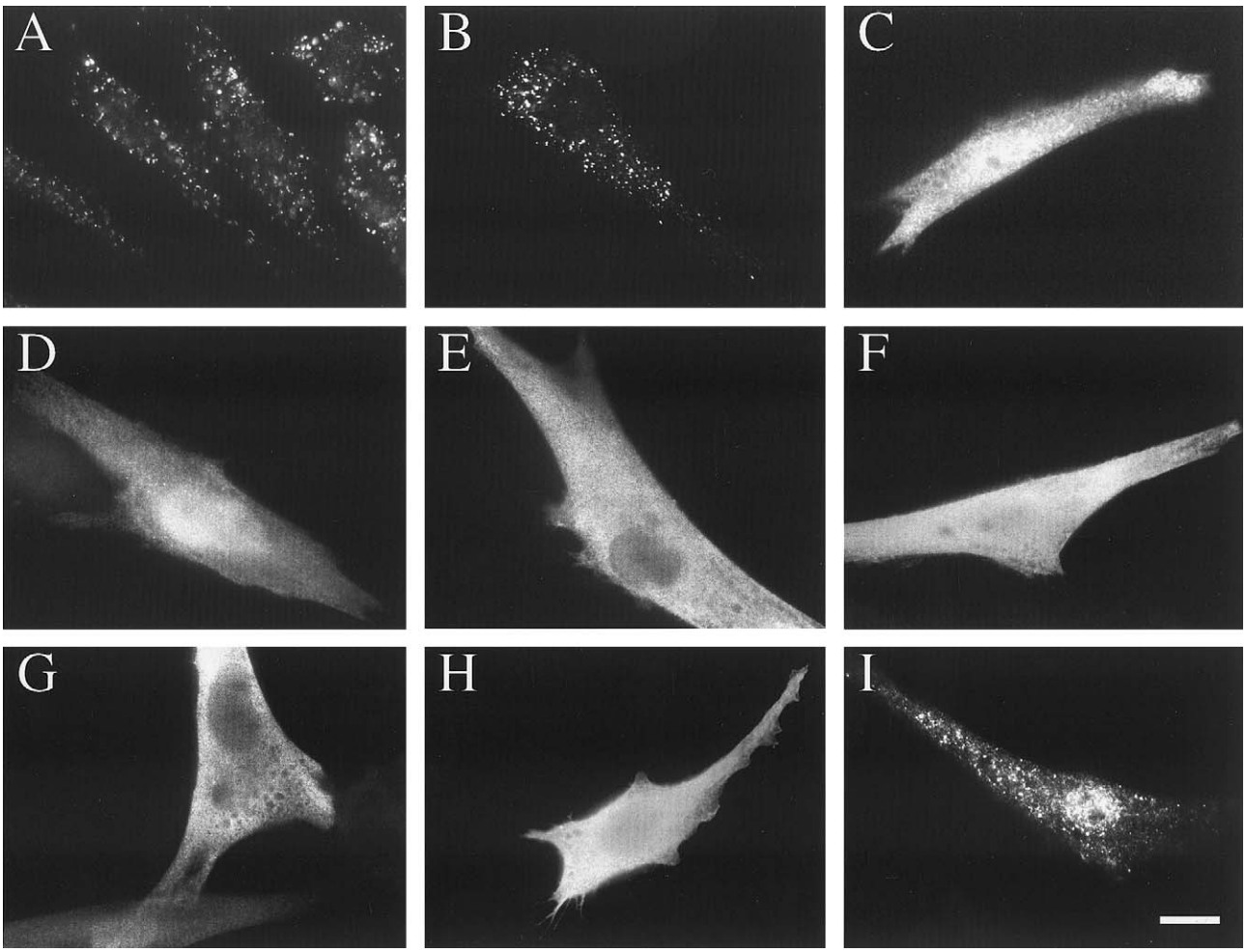

Figure 1 Peroxisomal protein–import properties of patient PBD222 cells. The human skin–fibroblast cell line from patient PBD222 was processed for single-label indirect immunofluorescence with rabbit antibodies specific for PMP70 (*A*), PEX14 (*B*), and fluorescein-labeled goat antirabbit secondary antibodies. These cells were also processed for immunofluorescence with sheep antibodies specific for catalase (*C*) and Texas red-labeled goat antisheep antibodies. Patient PBD222 cells were transfected with plasmids designed to express the peroxisomal marker proteins NmycCatalase (*D*), Nmyc PTE1 (*E*), NmycPECI (*F*), NmycGOX (*G*), and PAHXmyc (*H*) and processed for immunofluorescence two days later with mouse monoclonal anti-myc antibodies and Texas red-labeled goat antimouse secondary antibodies. These proteins are normally imported into the peroxisome lumen (Mihalik et al. 1997; Geisbrecht et al. 1999; J. M. Jones and S. J. Gould, unpublished data; Jones et al. 1999). Patient PBD222 cells were also processed for single-label immunofluorescence with rabbit anti-SKL antibodies (*I*), which recognize multiple PTS1-containing proteins (Gould et al. 1990), and fluorescein-labeled goat antirabbit secondary antibodies. Images were captured by fluorescence microscopy on an Olympus BH2 fluorescence microscope and Kodak TMAX400 film. Bar = 15 μm.

SKL_{COOH}, the canonical type 1 peroxisomal targeting signal: peroxisomal thioesterase (PTE1; Jones et al. 1999) and peroxisomal Δ^3,Δ^2 enoyl-CoA isomerase (PECI; Geisbrecht et al. 1999). We also examined the distribution of human peroxisomal glycolate peroxidase, which terminates in the atypical PTS1, SKI (GOX; J. M. Jones and S. J. Gould, unpublished data). Nmyc-tagged forms of all three proteins (NmycPTE1, NmycPECI, and NmycGOX) were predominantly cytoplasmic in patient PBD222 cells (fig. 1*E–G*) although they are predominantly peroxisomal in normal human fibroblasts. A myc-tagged version of the PTS2-marker protein, phytanoyl-CoA α-hydroxylase (PAHXmyc), which is efficiently targeted to peroxisomes in normal human fibroblasts (Mihalik et al. 1997), was also predominantly cytoplasmic in patient PBD222 cells (fig. 1*H*), demonstrating that the protein-import defect of these cells also applies to PTS2-targeted peroxisomal matrix proteins.

Given that patient PBD222 cells were derived from a patient mildly affected with NALD, we would expect that these cells would be able to import residual levels of peroxisomal matrix proteins. Consistent with this prediction, we did observe some peroxisomal import of the peroxisomal marker proteins in ~5%–15% of the patient PBD222 cells that were transfected with expression vectors for NmycPTE1, NmycPECI, and PAHXmyc (data not shown). Patient PBD222 cells showed no import of NmycCatalase and imported detectable levels of NmycGOX in <1% of expressing cells (data not shown). The residual level of peroxisomal matrix–protein import in patient PBD222 cells can also be visualized with an anti-SKL_{COOH} antibody that recognizes multiple PTS1-containing proteins. These antibodies detected the import of at least some peroxisomal matrix proteins in many patient PBD222 cells (fig. 1*I*).

PEX13 *Is Defective in Patient PBD222*

To identify the gene that is responsible for disease in CG13 of the PBDs, we transfected patient PBD222 fibroblasts with pcDNA3-based plasmids that are designed to express all known human *PEX* genes, including *PEX1* (Reuber et al. 1997), *PEX2* (Shimozawa et al. 1992), *PEX3* (Kammerer et al. 1998), *PEX5* (Dodt et al. 1995), *PEX6* (Yahraus et al. 1996), *PEX7* (Braverman et al. 1997), *PEX10* (Warren et al. 1998), *PEX11α* and *PEX11β* (Schrader et al. 1998), *PEX12* (Chang et al. 1997), *PEX13* (Bjorkman et al. 1998), *PEX14* (Fransen et al. 1998), *PEX16* (South and Gould 1999), and *PEX19* (Braun et al. 1994), as well as the control vector, pcDNA3. Two days after transfection, these cells were processed for indirect immunofluorescence with antibodies specific for human catalase, and the subcellular distribution of catalase in each transfected population was determined by visual inspection. We observed that only expression of *PEX13* rescued catalase import in patient PBD222 cells (table 2 and fig. 2).

To determine whether the expression of *PEX13* could also restore the import of other peroxisomal matrix proteins, we cotransfected patient PBD222 cells with pcDNA3 or with pcDNA3-*PEX13* together with plasmids designed to express PAHXmyc, NmycGOX, NmycPTE1, NmycPECI, and NmycCatalase. Two days after transfection, the cells were processed for indirect immunofluorescence with antibodies specific for the myc epitope tag and for PMP70, a marker of the peroxisome membrane. In all instances, cotransfection with the *PEX13* expression vector resulted in significantly greater import than occurred in cells transfected with vector alone (table 3). The association of these proteins with peroxisomes was demonstrated by their colocalization with PMP70 (fig. 3). Differential permeabilization experiments confirmed that the rescue observed in these cells represented import into the peroxisome lumen (data not shown).

These functional complementation studies provide strong evidence that defects in *PEX13* were the probable cause of disease in patient PBD222. To test this hypothesis we sequenced the *PEX13* gene from patient PBD222. The organization of the *PEX13* gene was recently elucidated (Bjorkman et al. 1998), allowing us to sequence all four coding exons of the *PEX13* gene from amplified fragments of genomic DNA. The sequence of the *PEX13* gene was determined both from patient PBD222 and from an unaffected individual. As expected, we detected a mutation in the *PEX13* gene from patient PBD222 (fig. 4). This mutation, a substitution of a threonine codon for the isoleucine 326 codon, is referred to as the I326T mutation. Patient PBD222 appears to be homozygous for this mutation, because we were unable to detect the wild-type sequence in this region of the

Table 2

Ability of Different Human *PEX* Genes to Restore Peroxisomal Protein Import in Patient PBD222 Cells

Transfected Plasmid	Catalase Import
pcDNA3	−
pcDNA3-*PEX1*	−
pcDNA3-*PEX2*	−
pcDNA3-*PEX3*	−
pcDNA3-*PEX5*	−
pcDNA3-*PEX6*	−
pcDNA3-*PEX7*	−
pcDNA3-*PEX10*	−
pcDNA3-*PEX11α*	−
pcDNA3-*PEX11β*	−
pcDNA3-*PEX12*	−
pcDNA3-*PEX13*	+
pcDNA3-*PEX14*	−
pcDNA3-*PEX16*	−
pcDNA3-*PEX19*	−

gene. Furthermore, our analysis of *PEX13* cDNAs from patient PBD222 revealed the presence of only one product, that with the I326T mutation (data not shown). Isoleucine 326 lies within the SH3 domain of PEX13, a region of the protein that is required for function and has been implicated in interactions with other peroxins (Elgersma et al. 1996; Erdmann and Blobel 1996; Gould et al. 1996; Girzalsky et al. 1999). Furthermore, this residue is a conserved feature of PEX13 from a variety of species, with either isoleucine or valine at this position of the PEX13 protein from humans, nematodes (*Caenorhabditis elegans*), and the yeasts *P. pastoris* and *Saccharomyces cerevisiae* (Gould et al. 1996).

The I326T Mutation Attenuates PEX13 *Activity*

The hypothesis that the *PEX13*/I326T mutation is responsible for disease in patient PBD222, who is mildly affected with NALD, makes two predictions. First, it predicts that this mutation will reduce *PEX13* activity in vivo. Second, it predicts that this mutation will not eliminate *PEX13* activity in vivo, for if it did, patient PBD222 should have displayed the more severe phenotypes of ZS rather than the milder phenotypes of

Table 3

***PEX13* Expression Rescues the Import of Multiple PTS1- and PTS2-Containing Peroxisomal Marker Proteins in Patient PBD222 Cells**

	Peroxisomal Protein Import (%)	
Marker Protein	pcDNA3	pcDNA3-*PEX13*
NmycCatalase	0/561 (0)	335/539 (62)
NmycGOX	4/1,081 (.4)	751/1,143 (66)
NmycPTE1	68/534 (13)	444/665 (67)
NmycPECI	77/565 (14)	373/505 (74)
PAHXmyc	11/659 (2)	444/626 (71)
Endogenous catalase[a]	17/543 (3)	130/587 (22)

[a] These numbers are not corrected for transfection efficiency.

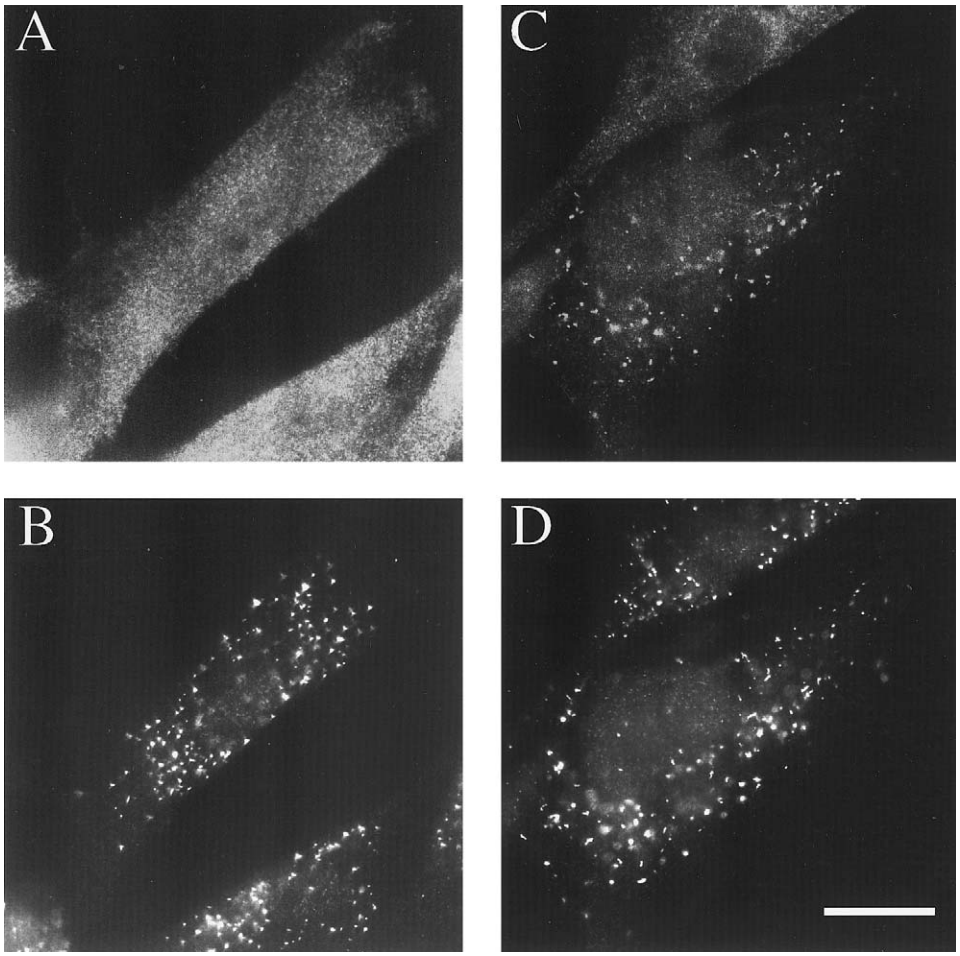

Figure 2 Catalase is imported into peroxisomes of patient PBD222 after expression of *PEX13*. Patient PBD222 cells were transfected with pcDNA3 (*A* and *B*) or pcDNA3-*PEX13* (*C* and *D*). Two days after transfection, the cells were processed for indirect immunofluorescence with sheep antibodies specific for human catalase (*A* and *C*) and rabbit antibodies specific for human PMP70 (*B* and *D*), followed by fluorescein-labeled goat antisheep and Texas red-labeled goat antirabbit secondary antibodies. Images were captured by fluorescence microscopy on an Olympus BH2 fluorescence microscope and Kodak TMAX400 film. Bar = 15 μm.

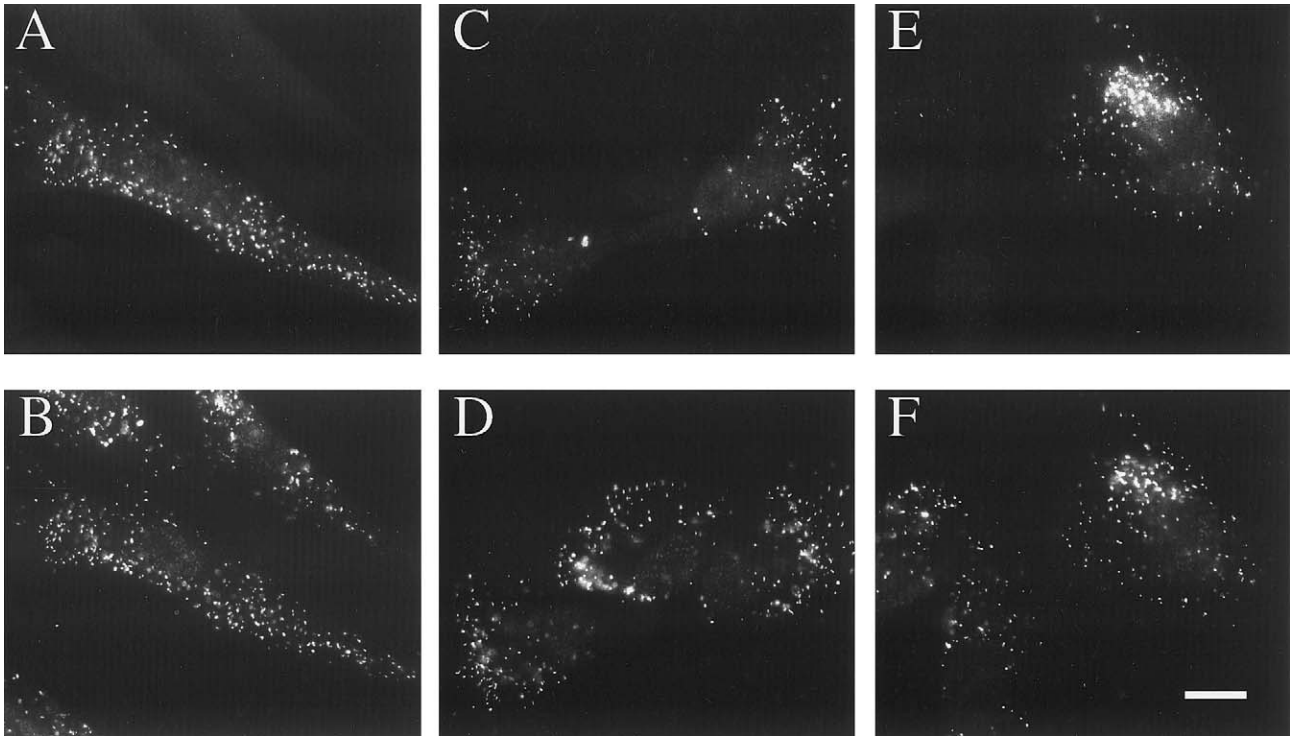

Figure 3 *PEX13* restores the import of multiple peroxisomal enzymes in patient PBD222 cells. Patient PBD222 cells were transfected with pcDNA3-*PEX13* and vectors designed to express myc-tagged versions of peroxisomal phytanoyl-CoA a-hydroxylase (*A* and *B*), glycolate oxidase (*C* and *D*), and catalase (*E* and *F*) (pcDNA3-PAHXmyc, pcDNA3-NmycGOX, and pcDNA3-NmycCatalase, respectively). Two days after transfection, the cells were processed for immunofluorescence with a mouse monoclonal antibody to the myc-epitope tag (*A, C,* and *E*) and a rabbit antibody specific for PMP70 (*B, D,* and *F*), followed by fluorescein-labeled goat antimouse and Texas red-labeled goat antirabbit secondary antibodies. Images were captured by fluorescence microscopy on an Olympus BH2 fluorescence microscope and Kodak TMAX400 film. Bar = 15 μm.

NALD. To test these predictions, we engineered the I326T mutation into the *PEX13* cDNA and assessed its effects on *PEX13* activity in vivo by using a functional complementation assay.

Patient PBD222 cells were cotransfected with (1) the plasmids pcDNA3, pcDNA3-*PEX13,* or pcDNA3-*PEX13*/I326T, and (2) plasmids designed to express myc-tagged versions of several human peroxisomal matrix enzymes (PAHXmyc, NmycGOX, NmycPTE1, NmycPECI, and NmycCatalase). Cells from each population were processed for immunofluorescence with antibodies specific for the myc tag, and cells expressing the marker proteins were scored on the basis of whether the marker protein was cytoplasmic only (fig. 5*A*), cytoplasmic and peroxisomal (fig. 5*B*), or peroxisomal only (fig. 5*C*). We also tested the ability of the wild-type and mutant *PEX13*-expression vectors to rescue the import of endogenously expressed catalase. The activity of the *PEX13*/I326T cDNA displayed a relative rescue activity with a range of 37%–85% of wild-type values, depending on the marker protein that was analyzed (table 4). Data for all trials are presented. These results demonstrate that the I326T mutation reduces but does not eliminate *PEX13* function, supporting the hypothesis that the I326T mutation is responsible for disease in patient PBD222.

An additional prediction from the mild phenotypes of patient PBD222 is that its mutation may be particularly poor at mediating complete import of peroxisomal proteins. In our calculation of relative rescue, we included cells that displayed either cytoplasmic and peroxisomal staining or peroxisomal only staining. We recalculated the rescue efficiencies for the *PEX13*- and *PEX13*/I326T–expression vectors by using the more strict criteria for import (peroxisomal only staining) to derive relative complete rescue. A comparison between these numbers and the relative rescue data suggest that the I326T mutation had a particularly severe effect on the ability of *PEX13* to restore full peroxisomal protein import in patient PBD222 cells, reducing its complete rescue to 2%–59%, relative to that of the wild-type *PEX13* expression vector (table 4).

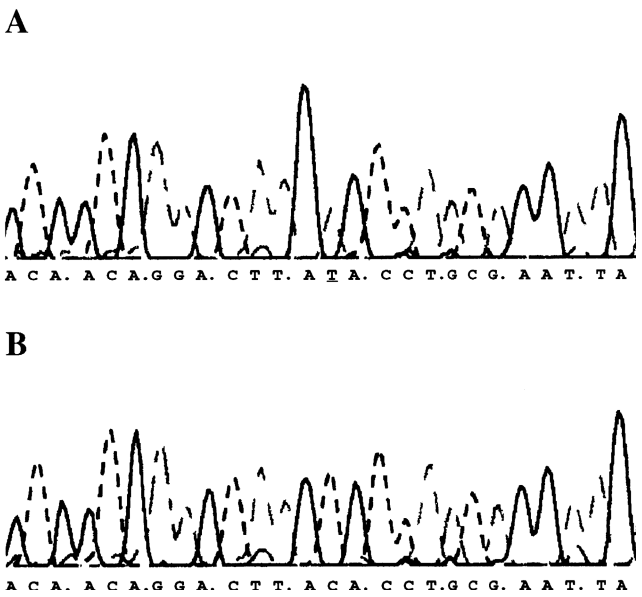

Figure 4 The *PEX13* mutation in patient PBD222. A genomic DNA fragment spanning the length of exon 4 was amplified from control and patient PBD222 genomic DNA and sequenced directly. The sequencing chromatogram revealed the presence of a single mutation in the fragment from patient PBD222 (*B*) that was not present in the control sample (*A*). This mutation changes the T of the 326 codon (underlined in *A*) to a C, resulting in the substitution of threonine for isoleucine at position 326 of the protein.

The Analogous Mutation in P. pastoris PEX13 *also Attenuates Activity*

We have demonstrated previously the utility of using yeast systems to study the effects of mutations in human *PEX* genes (Dodt et al. 1995). Given that I326 of human PEX13 is conserved as either an isoleucine or valine in PEX13 from a variety of organisms, including *P. pastoris,* we decided to test the effect of an analogous mutation in *P. pastoris PEX13*. The analogous residue in this protein is valine 334, and we engineered a V334T mutation into a *P. pastoris PEX13*-expression vector. Previous studies have established that *P. pastoris* utilizes peroxisomal enzymes for the metabolism of methanol and requires functional peroxisomes as a sole carbon source to grow on methanol (Gould et al. 1992). A *P. pastoris pex13*Δ strain was transformed with plasmids designed to express the wild-type *PEX13* gene (pDC120) and nonfunctional *PEX13* gene (pDC120/E291K) (Gould et al. 1996), as well as an analogous plasmid (pDC120/V334T) designed to express the *PEX13*/V334T gene. The growth, on methanol, of the resulting strains was then monitored over a period of several days (fig. 6). As previously demonstrated by Gould et al. (1996), pDC120 can complement the peroxisomal protein–import defects and growth defect of the *pex13*Δ strain, but the E291K mutation abrogates *PEX13* function. Interestingly, we observed that the strain carrying the *PEX13*/V334T–expression vector was able to grow on methanol, although with a greatly extended lag phase and reduced net growth. This intermediate phenotype suggested that the V334T reduced but did not eliminate *PEX13* function as observed for the I326T mutation in human *PEX13*.

The PEX13*/I326T Mutation Is Not a Common Polymorphism*

Although the preceding data provide strong evidence that the I326T mutation is responsible for disease in patient PBD222, an alternative hypothesis is that (1) a different gene is mutated in patient PBD222 cells, (2) one or both of the mutations in this putative gene are suppressed by transient overexpression of *PEX13* in our functional complementation assays, and (3) the *PEX13*/I326T mutation is a silent polymorphism unrelated to the disease. We have already noted that expression of other human *PEX* genes failed to rescue the phenotypes of patient PBD222 cells (table 2) and that the I326T mutation has a significant effect on *PEX13* activity. Nevertheless, we tested whether the *PEX13*/I326T mutation might be a polymorphism. We screened 102 *PEX13* genes from 51 control individuals for the presence of the I326T mutation. The *PEX13*/I326T mutation was not detected in any of the control genomic DNA samples (data not shown), suggesting that it is not a polymorphism.

Discussion

The initial report of patient PBD222 indicated that this patient displayed the relatively mild phenotypes typical of patients with NALD (Shimozawa et al. 1998). Here, we have examined the cellular phenotype of fibroblasts from this patient. On the basis of the import of the peroxisomal membrane proteins PEX14 and PMP70 we conclude that patient PBD222 cells do not display a severe defect either in peroxisome synthesis or in import of peroxisomal membrane proteins. However, we found that patient PBD222 cells were defective in import of all five peroxisomal matrix proteins that we tested. These included catalase, the most inefficiently imported peroxisomal matrix protein in mammalian cells (Lazarow et al. 1982). It is notable that catalase contains a variant form of PTS1, KANL (Purdue and Lazarow 1996), which may be responsible for its extremely inefficient import into peroxisomes. However, the peroxisomal protein–import defects in patient PBD222 were not restricted to catalase. We found that patient PBD222 cells also displayed defects in the import of peroxisomal thioesterase, peroxisomal Δ^3,Δ^2 enoyl-CoA isomerase, and peroxisomal glycolate oxidase (Geisbrecht et al.

1999; J. M. Jones and S. J. Gould, unpublished data; Jones et al. 1999). We also found that patient PBD222 cells were defective in the import of a PTS2-protein marker, phytanoyl-CoA α-hydroxylase (Mihalik et al. 1997). Thus, patient PBD222 cells displayed the typical phenotypes of NALD: partial defects in the import of PTS1- and PTS2-containing peroxisomal matrix proteins, but no apparent defect in the import of peroxisomal membrane proteins or in the synthesis of peroxisome membranes.

The phenotypes of patient PBD222 cells, like those of all patients with NALD, predict that the mutations that are responsible for disease will reduce but not eliminate the activity of the affected gene. We found that expression of *PEX13* restored peroxisomal protein import in patient PBD222 cells and that patient PBD222 was homozygous for a missense mutation, I326T, in the *PEX13* gene. This mutation attenuated but did not eliminate the ability of *PEX13* to rescue peroxisomal protein–import defects in patient PBD222 cells, a result that is consistent with the mild cellular and clinical phenotypes of patient PBD222. In addition, we engineered the analogous mutation (V334T) into the yeast *P. pastoris PEX13* gene and found that it caused a similar phenotype, reducing but not eliminating *PEX13* function.

These results strongly suggest that *PEX13* is the gene defective in patient PBD222 and complementation group 13 of the PBDs. However, it is necessary to consider the possibility that the *PEX13*/I326T mutation might be a silent polymorphism, and that the rescue of peroxisomal protein import in patient PBD222 cells by *PEX13* might represent an example of extragenic suppression, with the causative mutation lying in a different human *PEX* gene.

Table 4

The I326T Mutation Attenuates *PEX13* Activity

Marker Protein and Trial	Relative Rescue		Relative Complete Rescue	
	WT	I326T	WT	I326T
NmycCatalase:				
Trial 1	100	37	100	8
Trial 2	100	38	100	2
Trial 3	100	62	100	18
NmycGOX:				
Trial 1	100	65	100	39
Trial 2	100	58	100	37
Trial 3	100	71	100	59
NmycPTE1	100	77	100	45
NmycPECI	100	84	100	56
PAHXmyc:				
Trial 1	100	85	…	…
Trial 2	100	61	100	16
Endogenous catalase	100	43	…	…

Note.—Values given are percents.

However, several lines of evidence argue against this possibility. First, the I326T mutation was not detected in >100 control alleles that were assayed, demonstrating that it is not a common polymorphism. Second, we found that none of the other known human *PEX* genes (including *PEX1, PEX2, PEX3, PEX5, PEX6, PEX7, PEX10, PEX11α*, *PEX11β*, *PEX12, PEX14, PEX16,* and *PEX19*) were able to rescue peroxisomal protein import in patient PBD222 cells. It is particularly notable that none of the human homologues of yeast peroxins that interact with yeast PEX13 (i.e., PEX5, PEX7, and PEX14; see Elgersma et al. 1996; Erdmann and Blobel 1996; Gould et al. 1996; and Girzalsky et al. 1999) could

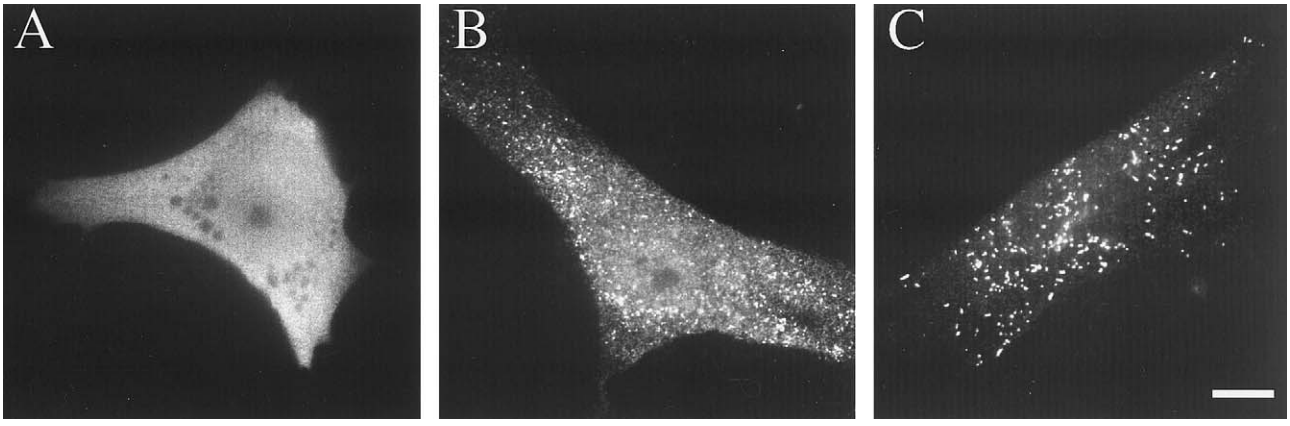

Figure 5 Different degrees of phenotypic rescue in functional complementation assays. Patient PBD222 cells were transfected with pcDNA3-*PEX13* and pcDNA3-NmycPECI. Two days later, these cells were processed for indirect immunofluorescence with a mouse monoclonal antibody to the myc-epitope tag and fluorescein-labeled goat antimouse secondary antibodies. The transfected cells displayed three basic phenotype: no import of NmycPECI (*A*), both cytoplasmic accumulation and peroxisomal import of NmycPECI (*B*), and predominantly peroxisomal staining for NmycPECI (*C*). Images were captured by fluorescence microscopy on an Olympus BH2 fluorescence microscope and Kodak TMAX400 film. Bar = 15 μm.

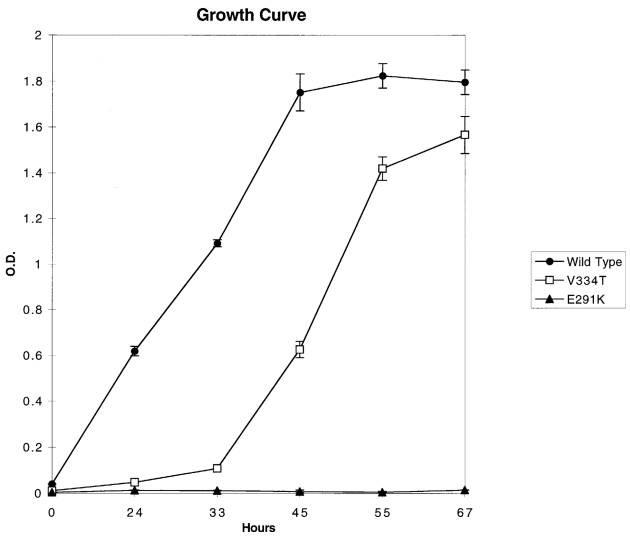

Figure 6 The V334T mutation in *P. pastoris PEX13* attenuates its activity in vivo. Growth curves in minimal-methanol medium for *P. pastoris pex13*Δ strains carrying the *PEX13*-expression plasmids pDC120 (wild-type; denoted by blackened circles), pDC120/E291K (inactive mutant; denoted by blackened triangles), and pDC120/V334T (denoted by unblackened squares). Data represent the average of three independent set of cultures, and the standard deviations are presented as brackets above and below data point.

rescue the import defects in patient PBD222 cells. Third, the I326T mutation significantly reduced *PEX13* activity, and an analogous mutation in the *P. pastoris PEX13* gene also reduced its activity. Finally, the efficiency with which *PEX13* rescued peroxisomal protein import in patient PBD222 cells was roughly equivalent to the efficiency with which we could introduce the *PEX13*-expression vector into patient PBD222 cells. In our previous report of extragenic suppression in the PBDs (between *PEX1* and *PEX6*), we observed that extragenic suppression was much less efficient (≤25%) than complementation with the gene that was mutated in the cells (Geisbrecht et al. 1998).

The high relative activity retained by the *PEX13*/I326T cDNA (40%–80%, depending on the marker protein being assayed) is not without precedent for mild alleles of human *PEX* genes. The common G843D mutation in *PEX1* retains ~20% of the complementing activity of wild-type *PEX1* (Reuber et al. 1997), and the H290Q mutation in *PEX10* retains 50%–85% complementing activity as compared with that of the wild-type *PEX10* gene (Warren et al. 1998). Furthermore, it should be noted that the activities reported for the *PEX13*/I326T cDNA were not determined in a *PEX13*-null cell line but in a cell line—that of patient PBD222—that retains some *PEX13* activity. In contrast, the high activities for the *PEX1*/G843D and *PEX10*/H290Q mutations were determined against a null background in cell lines that lacked any residual *PEX1* or *PEX10* activity, respectively. It should also be noted that there is no heterozygote phenotype in the PBDs, indicating that the amount of gene function required to generate full rescue may be significantly less than the full activity of the wild-type *PEX13* gene. As such, the relative rescue activities determined in our functional complementation assay may be significantly higher than the actual amount of gene function encoded by mutant alleles.

PEX13 was initially ruled out as the gene that is defective in patient PBD222 (Shimozawa et al. 1998). This conclusion was made on the basis of the observation that expression of a *PEX13* cDNA failed to rescue matrix-protein import in patient PBD222 cells. However, the *PEX13* cDNA that was expressed in the prior study by Shimozawa et al. (1998) corresponded to a *PEX13* cDNA that was missing exon 1 sequences and that encoded a truncated protein that lacked the N-terminal 39 amino acids of PEX13. Using our functional complementation assay, we observed that this truncation reduced *PEX13* activity to ~20% of wild type and may have interfered with the ability of Shimozawa et al. (1998) to detect rescue. However, the fact that the *PEX13* cDNA used in that study contained an unfavorable context for initiation at the first ATG of the ORF may also have been a contributing factor; specifically, the *PEX13* cDNA contained a T at the −3 position relative to the A of the start codon. Studies of efficiently translated mammalian mRNA indicate that the −3 position is almost always a purine (usually an A) and that its replacement with a pyrimidine can result in translation initiation at downstream start codons rather than at the first ATG of the ORF (Kozak 1992, 1996). If the protein product of the truncated *PEX13* cDNA used by Shimozawa et al. (1998) initiated at the next downstream ATG, then it would result in the loss of the N-terminal 145 amino acids of PEX13. We have previously found that large N-terminal deletions of ≥100 amino acids eliminated the function of *P. pastoris PEX13*, whereas smaller N-terminal deletions retained significant *PEX13* activity (Gould et al. 1996).

Our results provide strong evidence that the I326T mutation in *PEX13* is the cause of disease in patient PBD222. The next challenge is to determine how this mutation affects the role of PEX13 in peroxisome biogenesis. A variety of studies have established that the PTS1 and PTS2 receptors, PEX5 and PEX7, are predominantly cytoplasmic proteins that serve to shuttle newly synthesized peroxisomal matrix proteins from the cytoplasm to the peroxisome (Kunau and Erdmann 1998). However, it is also clear that there are detectable quantities of the PTS1 and PTS2 receptors associated with peroxisomes at steady state. PEX13, along with PEX14 and PEX17, has been implicated in the docking

of these receptors to the peroxisome membrane, and the SH3 domain of PEX13 has been implicated in binding PEX5 (Elgersma et al. 1996; Erdmann and Blobel 1996; Gould et al. 1996; Huhse et al. 1998; Girzalsky et al. 1999). The *PEX13* mutation I326T, identified here in patient PBD222, lies within the PEX13 SH3 domain and substitutes a polar residue, threonine, for the hydrophobic residue, isoleucine, that is normally found at this position. As such, the I326T mutation may be expected to affect the interaction between PEX13 and one or more of its interacting proteins. Molecular replacement modeling with the three-dimensional structure of the Fyn SH3 domain (Musacchio et al. 1994) used as the template allowed us to assess the possible structural effects of the I326T mutation. These modeling experiments suggest that I326 may interact with F293, a residue within the large RT-loop of the PEX13 SH3 domain, and that this interaction may stabilize the fold of the SH3 domain. Replacement of I326 with the polar threonine residue is predicted to disrupt this interaction with F293, with one possible result being the destabilization of the RT-loop—a region of the protein involved in substrate recognition. Additional experiments to determine the effects of this mutation on the interaction between PEX13 and its partner proteins should provide important insight into why the I326T mutation disrupts peroxisomal protein import.

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